Published in final edited form as:

Environ Int. 2018 November ; 120: 373–381. doi:10.1016/j.envint.2018.08.010.

Intrauterine Multi-Metal Exposure is Associated with Reduced Fetal Growth through Modulation of the Placental Gene Network.

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Abstract

Background.—Intrauterine metal exposures and aberrations in placental processes are known contributors to being born small for gestational age (SGA). However, studies to date have largely focused on independent effects, failing to account for potential interdependence among these markers.

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Declarations of interest: none

Objectives.—We evaluated the inter-relationship between multi-metal indices and placental gene network modules related to SGA status to highlight potential molecular pathways through which *in utero* multi-metal exposure impacts fetal growth.

Methods.—Weighted quantile sum (WQS) regression was performed using a panel of 16 trace metals measured in post-partum maternal toe nails collected from the Rhode Island Child Health Study (RICHs, n=195), and confirmation of the derived SGA-related multi-metal index was conducted using Bayesian kernel machine regression (BKMR). We leveraged existing placental weighted gene coexpression network data to examine associations between the SGA multi-metal index and placental gene expression. Expression of select genes were assessed using RT-PCR in an independent birth cohort, the New Hampshire Birth Cohort Study (NHBCS, n=237).

Results.—We identified a multi-metal index, predominated by arsenic (As) and cadmium (Cd), that was positively associated with SGA status (Odds ratio = 2.73 [1.04, 7.18]). This index was also associated with the expression of placental gene modules involved in “gene expression” ($\beta = -0.02$ [-0.04, -0.01]) and “metabolic hormone secretion” ($\beta = 0.02$ [0.00, 0.05]). We validated the association between cadmium exposure and the expression of *GRHL1* and *INHBA*, genes in the “metabolic hormone secretion” module, in NHBCS.

Conclusion.—We present a novel approach that integrates the application of advanced bioinformatics and biostatistics methods to delineate potential placental pathways through which trace metal exposures impact fetal growth.

Keywords

Multi-metal exposure; placenta; gene coexpression network; birth weight

Introduction

Being born small for gestational age (SGA) is a major determinant of childhood and later life morbidity, including metabolic syndrome, neurodevelopmental deficits and coronary heart disease (Arcangeli et al. 2012; Jancevska et al. 2012). Established risk factors known to impact fetal growth include maternal age, parity and ethnicity (Jancevska et al. 2012). In addition to maternal characteristics, gestational exposure to environmental pollutants through maternal ingestion and inhalation are also known to play a role (Chou et al. 2011; Lauritzen et al. 2016; Peelen et al. 2016; Stillerman et al. 2008). Multiple studies to date have linked intrauterine trace metal levels to SGA status. These include exposure to elevated levels of toxic metals (i.e., arsenic (Claus Henn et al. 2016; Thomas et al. 2015), cadmium (Cheng et al. 2017; Johnston et al. 2014; Sun et al. 2014) and lead (Nishioka et al. 2014; Taylor et al. 2015)), reduced levels of essential elements (i.e., copper, zinc and iron (Shen et al. 2015)) and several studies demonstrating curvilinear associations (i.e., manganese (Chen et al. 2014; Xia et al. 2016)). However, inconsistencies in the literature persist (Bermúdez et al. 2015; Loiacono et al. 1992; Osman et al. 2000; Thomas et al. 2015).

While heterogeneity in study designs likely plays an important role, the discrepancy may also reflect a focus on assessing the effects of individual metals. Such methods fail to account for potential mixture compositions in which the presence of toxic and essential co-pollutants at varying doses may alter the activity of the metal under consideration. While

findings are beginning to emerge demonstrating modified effects within the context of two metals at a time (Al-Saleh et al. 2015; Everson et al. 2017), the role of the multi-metal environment on deviations of appropriate fetal growth is still underexplored.

The molecular pathways through which metals exert their effect on fetal growth are not clearly delineated. However, several studies point to the possibility that in utero exposure to metals at toxic levels may induce aberrations in processes mediated by the placenta, the organ overseeing appropriate fetal development (Gundacker and Hengstschläger 2012). Alterations in the gene expression and DNA methylation profile of several placental loci, including genes involved in nutrient transport, endocrine signaling and imprinting, have been linked to fetal growth (Caviedes et al. 2016; Chen et al. 2015; Green et al. 2015; Kappil et al. 2015; Lesseur et al. 2013; Sabri et al. 2014). However, similar to studies linking trace metals to fetal growth, molecular biomarker studies thus far have focused on associations between individual genes and fetal growth. As biological processes are driven by interacting gene-sets, testing the independent association of individual genes likely results in information loss on the biologic context within which perturbations occur. In an effort to address the co-regulated organizational structure of genes, we recently delineated the human placental coexpression network and demonstrated deviations in specific network modules linked to aberrant fetal growth (Deyssenroth et al. 2017).

Similar to the bioinformatics methods developed to analyze genes within network contexts, novel statistical approaches that are able to model and delineate the independent and joint effects across multiple correlated exposures, are now available to address the gap in the literature regarding exposure response relationships (Liu et al. 2017; Stafoggia et al. 2017). These include weighted quantile sum (WQS) regression (Carrico et al. 2014) and Bayesian kernel machine regression (BKMR) (Bobb et al. 2015). While the exposure-response relationship modeled by the WQS-derived body burden index is constrained to linear, unidirectional associations, the machine learning based BKMR method allows more flexible modeling of the relationship between co-pollutants and the outcome. The former approach lends itself for enhanced interpretability of the findings while the latter approach allows for more in-depth evaluation of potentially complex, non-linear and non-additive exposure-response relationships.

In the current study, we integrate the application of novel biostatistics and bioinformatics approaches to identify an SGA-related multi-metal index and assess whether SGA-related placental gene networks are associated with this multi-metal index to highlight potential molecular pathways through which *in utero* trace metal exposure impacts fetal growth.

Materials and Methods

Study participants

Mother-infant pairs were enrolled in the Rhode Island Child Health Study between 2009–2013, following delivery at Women and Infants Hospital (n=899) (Kappil et al. 2015). Enrollment was restricted to mothers ≥18 years of age and infants without congenital or chromosomal abnormalities. Infants born small for gestational age (SGA, <10% percentile) and large for gestational age (LGA, >90% percentile), based on the sex-specific actual-age

2013 Fenton Growth Chart (Fenton and Kim 2013), were matched on gender, gestational age and maternal age to infants born appropriate for gestational age (AGA). Anthropometric and clinical in-patient data were collected through structured reviews of medical records. Interviewer-based questionnaires were administered after delivery and prior to hospital discharge to collect demographic characteristics and exposure histories. Written informed consent was obtained from all enrolled participants, and the study was approved by the institutional review boards at Women and Infants Hospital and Emory University. The current study included SGA and AGA infants with complete molecular profile (placental RNA-Seq) and metal exposure data (n=195).

Findings relating metal exposure to placental gene expression were validated in an independent cohort, the New Hampshire Birth Cohort Study (NHBCS), among SGA and AGA participants with available extracted placental RNA (n=237). The NHBCS is a prospective birth cohort initially designed to assess the impact of intrauterine environmental exposures on child health and development. Participants were recruited from prenatal clinics in New Hampshire starting in 2009 (Emond et al. 2018). Similar to the RICHs cohort, available data in NHBCS includes Fenton growth curve measurements and metal levels assessed in maternal postpartum toenails. In contrast to RICHs, the NHBCS cohort was not oversampled for extreme birth weight groups and is therefore more reflective of growth distributions observed in the general population (Supplemental Table 1).

Metal assessment

First toenail clippings were requested from mothers and infants following discharge, and clippings were mailed back in provided envelopes. In RICHs, average time to collection was 2.8 months (range, 0.3–7.1 months) postpartum, while in NHBCS, all toenails were collected within 2–8 weeks postpartum. A panel of nineteen trace metals (silver (Ag), aluminum (Al), arsenic (As), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), mercury (Hg), manganese (Mn), molybdenum (Mo), nickel (Ni), lead (Pb), antimony (Sb), selenium (Se), tin (Sn), uranium (U), vanadium (V), and zinc (Zn)) were analyzed at the Dartmouth Trace Element Analysis laboratory using standardized ICP-MS protocols. Briefly, visible dirt was manually removed from toenail samples and toenails were further cleaned with five washes in an ultrasonic bath using Triton X-100 and acetone followed by deionized water. Toenails were allowed to dry prior to low-pressure microwave digestion and ICP-MS analysis. Quality control measures included the use of certified reference materials (Japanese hair standard NIES #13), analytical duplicates and spikes, initial and continuing calibration verification and digestion of fortified blanks (Punshon et al. 2016; White et al. 2018). The current study focused on the measurements derived from maternal toenails. Three metals with greater than 10% missing values (values below limit of detection (LOD) that fell below calibration blank), Ag, Co and Hg, were excluded from the analysis to maintain adequate sample size. Remaining measurement values falling below the LOD were replaced by the LOD/ 2.

Placental Gene Network

A placental gene coexpression network consisting of 17 network modules was generated from available placental RNA-Seq data using the “WGCNA: weighted correlation network

analysis” package in R as previously described (Deyssenroth et al. 2017). The first principal component of each module, the module eigengene, was derived as an average measure of module gene expression.

Quantitative real time PCR (qRT-PCR) validation

Genes from arsenic (*C21orf91*, *KIAA1370*, *INO80D*) and cadmium (*GRHL1*, *INHBA*, *LEP*) responsive placental network modules were selected for qRT-PCR verification in an independent cohort (NHBCS) based on connectivity within the module ($kME > 0.8$), association with the derived WQS index and availability of published PCR assays. In a preliminary study of 30 NHBCS participants (15 participants in the top quartile of exposure (As and Cd) and 15 participants in the lowest quartile of exposure (As and Cd), we did not observe differential expression among the As-responsive genes and As exposure while a trend towards significance was observed in the association between expression of the Cd-responsive genes and Cd exposure (data not shown). Based on these preliminary findings and due to limited RNA availability, the remainder of the NHBCS cohort was analyzed using the Cd-responsive genes. Placental RNA samples were reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) following manufacturer’s protocol. Based on an evaluation of the RICHs RNAseq data for a panel of commonly utilized housekeeping genes (*GAPDH*, *ACTB*, *SDHD*, *RPL19*, *RPL0* and *UBQ*), *RPL19* demonstrated the lowest coefficient of variation across the placental samples and was selected as the endogenous control gene in the current study. All samples were run in triplicate, alongside non-template controls and pooled internal controls for each assay (*GRHL1*, *INHBA*, *LEP* and *RPL19*) on each plate. Previously published primer sequences were utilized for *INHBA* (Katayama et al. 2017) and *LEP* (Haffeejee et al. 2017), and all remaining assays were designed using the NCBI Primer-BLAST tool (Supplemental Table 2).

The qRT-PCR reactions were conducted on a LightCycler 480 II Instrument (Roche Diagnostics Corporation, Indianapolis, IN) using the following thermocycling conditions: an initial denaturation step at 95°C for 10min, followed by 45 cycles at 95°C for 30sec, 60°C at 30 sec and 72°C at 30sec.

Statistical Analysis

Differences in maternal-infant demographic and gestational characteristics across birth weight categories and study participants were assessed using a Mann-Whitney U Test for continuous variables and a chi-square test for categorical variables.

The association between exposure to individual metals (log2 transformed) and SGA status was assessed using logistic regression models. To account for the collinearity among metals, weighted quantile sum (WQS) regression analysis was conducted to derive metal mixture indices associated with SGA status using the “gWQS: generalized weighted quantile sum regression” package in R (Carrico et al. 2014; Czarnota et al. 2015; Renzetti et al. 2016). The weights assigned to the individual, tertile-scored metals within the composite index were simultaneously derived based on bootstrap sampling ($n=200$), with the finalized weighted quantile score (WQS) estimated as the mean of the estimated estimates across the

bootstrap samples. Two separate WQS indices were derived, an index positively associated with SGA status and an index negatively associated with SGA status, each of which were separately assessed in association with fetal growth restriction using logistic regression models.

To assess the robustness of the major drivers of SGA status identified in our WQS analysis, we repeated the mixture modeling analysis based on Bayesian kernel machine regression (BKMR) using the “bkmr” package in R (Bobb 2017). In BKMR, the unknown exposure response relationship is specified using a kernel function. In our analysis, we applied a Gaussian kernel as previously described (Bobb et al. 2015). The Gaussian kernel can be considered as a similarity metric, which quantifies the distance, or the similarity, between two subjects’ exposures using Euclidean distance. Our final model was fit by running the Markov chain Monte Carlo (MCMC) sampler for 20,000 iterations. Because the Markov chain initiates from an arbitrary starting point, we discarded the initial portion of the chain (the first 10,000 iterations) as burn-in, to reduce the impact of starting values on the posterior inference. This allows us to conduct posterior inference using the later portion of the chain which has converged to the stationary distribution. In addition to log₂ transformation of the metals, all independent variables, including metals and covariates, were centered and scaled.

The association between network module eigengenes and SGA status was additionally assessed using logistic regression models. Generalized linear regression models were conducted to assess the association between SGA-associated metals (individual and WQS composite index) and SGA-associated network module eigengenes.

We also assessed gene-metal associations in an in silico analysis, interrogating the Comparative Toxicogenomics Database (Davis et al. 2017) for all gene associations reported for the 16 trace metals included in our analysis. The placental gene network was crossreferenced against this curated list of known gene-metal interactions to identify modules enriched for metal-responsive genes using a Fisher’s exact test (FDR<0.25). NHBCS qRT-PCR data were preprocessed and analyzed as follows. Cycle threshold (C_t) values ≥ 35 cycles were considered non-detectable. For each assay, samples with ≥ 2 nondetectable measurements were removed from the analysis, leaving a sample size of 262, 267 and 260 for the *GRHL1*, *INHBA* and *LEP* assays, respectively. Among these participants with placental expression data on *GRHL1*, *INHBA* and *LEP*, maternal toenail Cd levels were available for 180, 178, and 176 subjects, respectively. Triplicate C_t values for each sample/assay were averaged, and C_t values for each sample/assay were calculated using the following formula: $\bar{C}_t(RPL19) - \bar{C}_t(\text{Target})$. Generalized linear models were evaluated assessing the association between maternal toenail Cd levels and expression of the three selected genes.

In the RICHs dataset, all regression models were adjusted for infant gender, maternal ethnicity, maternal BMI and maternal smoking status during pregnancy based on observed differences in these variables across birth weight categories ($p < 0.20$). Metal analyses were additionally adjusted for metal assay batch. For the qRT-PCR evaluation among the NHBCS

samples, models were adjusted for PCR plate batch, infant gender, maternal BMI and maternal ever smoking status (>95% of women reported white ethnicity).

All analyses were conducted using R version 3.3.1

Results

The maternal-infant demographic and gestational characteristics among RICHs study participants are shown in Table 1. On average, birth weight was lower among SGA infants than AGA infants, as expected. A greater proportion of non-Caucasian women gave birth to SGA infants than Caucasian women. Additionally, a higher proportion of SGA infants were born among women who reported smoking during pregnancy than those who did not. No other clear differences in maternal-infant characteristics were observed across the two birth weight categories. The SGA/AGA subset of the RICHs cohort with available trace metal data differed from those without available trace metal data (Supplemental Table 3). Women who provided postpartum toenails were older, attained higher educational status and were less likely to smoke than women who did not provide postpartum toenails. The role of socioeconomic status in study retention observed in our study is in line with previous reports in longitudinal cohort studies (Launes et al. 2014).

The distribution of maternal metal levels assessed in RICHs study participants is shown in Table 2. Essential metals, including Zn, Fe, and Se, were detected at elevated levels compared to known toxicants, including Pb, As and Cd.

As shown in Figure 1, logistic regression models of individual metals and fetal growth restriction indicated borderline increases in the odds of SGA status with increasing As levels (OR=1.55, 95% CI [0.96,2.50]) and Cd levels (OR=1.37, 95% CI [0.96,2.00]) and a decrease in the odds of SGA status with increasing Ni levels (OR=0.78, 95% CI [0.65,0.94]) that reached statistical significance. In our WQS analysis, we observed a 2.7 increase in the odds of SGA status (OR = 2.73, 95% CI [1.04, 7.18]) for every unit increase in the derived metal mixture index (which represents tertiled metals, each weighted based on its contribution to the overall association with the outcome). The weights of As (44.4%) and Cd (17.8%) predominated in this metal mixture index, indicating their variable importance in driving the observed association. Similarly, a metal mixture index predominated by Ni (23.6%) and Al (22.3%) was significantly associated with decreased odds of SGA status (OR = 0.24, 95% CI [0.08, 0.76]) (Figure 2). These exposure-outcome relationships were corroborated using BKMR. Here, each metal's association to the outcome, with all other metals held at the median, also indicated that SGA status was positively associated with As and Cd and inversely associated with nickel (Supplemental Figure 1). This profile also suggests that As, Cd and Ni are linearly related to SGA status. Further evaluation of bivariate relationships among the metals revealed no interactive relationships among the metals within the BKMR-derived multi-metal index (Supplemental Figure 2).

We previously characterized the placental gene coexpression network from transcriptome-wide RNASeq data assessed in the same subset of RICHs study participants, and we reported 5 out of 17 derived gene network modules that demonstrated differential module

activity across 3 birth weight categories (small, appropriate, and large for gestational age) based on a crude ANOVA analysis (Deyssenroth et al. 2017). Building off these reported findings, we performed an adjusted logistic regression analysis modeling the eigengenes of the 17 derived placental network modules, contrasting SGA and AGA infants. Two of the five previously reported modules maintained a significant association in this more focused analysis adjusted for covariates, with a significant inverse association between the “greenyellow” module and SGA status and a significant positive association between the “salmon” module and SGA status (Figure 3). The “greenyellow” placental network module consists of 201 genes and is functionally enriched for biological processes related to gene expression based on Gene Ontology analysis, and the “salmon” placental network module consists of 162 genes and is functionally enriched for biological processes related to metabolic hormone secretion (Deyssenroth et al. 2017).

Among the SGA-related modules, a significant inverse association between the gene expression (greenyellow) module and As exposure ($\beta = -0.02$ [$-0.04, -0.01$]) as well as the SGA-associated metal mixture index ($\beta = -0.04$ [$-0.07, -0.01$]) was sustained using a generalized linear regression model. Similarly, borderline positive associations between the metabolic hormone secretion (salmon) module and Cd exposure ($\beta = 0.01$ [$0.0, 0.03$]) as well as the SGA-associated positive WQS index ($\beta = 0.02$ [$0.00, 0.05$]) was observed (Figure 4). A separate analysis based on mapping a curated list of known gene-metal interactions from the Comparative

Toxicogenomics Database (CTD) (Davis et al. 2017) onto our placental gene network revealed an enrichment of metal-responsive genes in several network modules, including a moderate, significant fold enrichment of As-responsive genes in the gene expression (greenyellow) module (OR=1.39 [$0.99, 1.92$], Supplemental Figure 3). A connectivity map of the gene expression (greenyellow) module highlighting the 50 As-responsive genes mapped from the CTD is shown in Figure 5. This figure also highlights several highly interconnected genes within this module.

These hub genes likely drive a substantial proportion of the variability in the eigengene and are, therefore, probable master regulators of the biological processes linked to this module. As shown in Supplemental Figure 4, 4 of the top 10 most highly connected green expression (greenyellow) module hub genes (*ANKRD12*, *C21orf91*, *FAM122B* and *INO80D*) were inversely associated with SGA status. These genes were also inversely associated with As exposure and/or the WQS index. Four of the top 10 most highly connected metabolic hormone secretion (salmon) module hub genes (*C8orf58*, *GRHL1*, *NDRG1* and *PVRL4*) are associated with SGA status. Out of these genes, *GRHL1* was additionally associated with Cd exposure (Supplemental Figure 5).

To assess the robustness of our findings, we evaluated the observed association between Cd exposure and three metabolic hormone secretion (salmon) module genes in NHBCS, an independent birth cohort. Generalized linear models indicated a positive direction in the relationship between maternal Cd levels and C_t values of the three analyzed genes, with the observed associations reaching statistical significance for *GRHL1* and *INHBA* ($p < 0.05$). Here, C_t values are operationalized so that increasing values indicate increasing expression

levels. Hence, our observations in NHBCS are consistent with the relationship between these metabolic hormone secretion (salmon) module genes and maternal Cd exposure observed in RICHs (Figure 6).

Discussion

In this study, we identified a multi-metal index predominated by As and Cd that is associated with SGA status. While previous studies have shown independent associations between fetal growth restriction and As as well as Cd exposure, this is the first study to demonstrate that the effect of these metals persist and predominate even after accounting for the presence of correlated co-pollutants. The identification of As and Cd as the predominant SGA-related bad actors among the evaluated panel of metals was substantiated with BKMR, an independent mixture modeling approach. Furthermore, this metal signature was also associated with SGArelevant placental gene network modules that are enriched for biological processes related to gene expression and metabolic hormone secretion, implicating potential molecular pathways through which these environmental contaminants exert their intrauterine effect.

Of note, most trace metal levels detected in RICHs participants are on par with reported levels of these metals in other US populations (Slotnick et al. 2005). The observed levels of the predominant metals associated with SGA status in our study, As (mean=0.049ug/g, range=0.012–0.445ug/g) and Cd (mean=0.016ug/g, range=0.001–0.170ug/g), are, in fact, lower than what is reported in other populations (Slotnick et al. 2005). Notably, while guidelines for acceptable Cd levels measured in nails are not established, the Agency for Toxic Substances and Disease Registry (ATSDR) lists As levels detected below 1ppm in nails as within the normal range. In our study, all individuals fell well below this stipulated set-point, suggesting that adverse health effects are observable at what is currently accepted as safe human exposure levels. On a broader scale, our findings suggest that accounting for co-pollutant exposures may reveal lower ‘safe level’ thresholds than what is demonstrated by evaluating exposures in isolation.

We identified two SGA-associated placental network modules associated with the multi-metal index. The As/Cd responsiveness of several genes within these modules were previously described. For example, changes in the expression of loci mapped to the gene expression (greenyellow) module, including *PAN3* (Yamagishi et al. 2014), *SUZ12* (Kim et al. 2012) and a panel of ZNF genes (Severson et al. 2013), were reported in relation to As exposure. Similarly, loci mapped to the metabolic hormone secretion (salmon) module, including *ARNT2* (Kluxen et al. 2012) and *INHBA* (Garrett et al. 2013), are known Cd-responsive genes. To identify metalresponsive genes in our placental gene network on a more comprehensive scale, we mapped studies indexed in the Toxicogenomics database that evaluated metals included in our study onto our placental gene network. Several of the observed enrichments aligned with the biological processes linked to the modules. For example, Fe is enriched in the “grey60” module, which predominates in heme-related genes involved in gas exchange. This analysis also indicated that the gene expression (greenyellow) module is enriched with As-responsive genes. In addition to corroborating previous reports of genes independently associated with As and Cd exposure, our findings

on a gene-network level suggest that many of these genes participate in common pathways (i.e., gene expression and metabolic hormone secretion) that may be dysregulated upon exposure.

Both the metal and gene signatures were derived while accounting for the correlated and interactive components in each data-set, allowing us to derive the predominant actors within the respective metal and genetic contexts. Furthermore, our WQS-derived multi-metal index was independently corroborated using the machine learning method, BKMR. While WQS stipulates an additive relationship among the constituents in the mixture in relation to the outcomes, BKMR allows for complex interactions among components of the exposure mixture, and potentially non-linear and non-additive associations between co-pollutant exposures and the health endpoint. This added level of granularity enabled us to verify that As and Cd are linearly related to the outcome and rule out potential multiplicative interactions among the analyzed metals. Additionally, we were able to confirm our expression-related findings in a select group of genes both in an independent cohort and using a different technological platform. Given that the NHBCS differs in several key demographic characteristics from the RICHs, most notably in terms of birth weight distributions and maternal ethnicity, the confirmatory analysis suggests our findings are generalizable beyond our study.

Important caveats warrant caution in the interpretation of the findings reported herein. The coexposure effect examined in the current study was limited to the available measurements of 16 trace metals. The maternal environment is likely influenced by a wider breadth of exogenous and endogenous factors, such as persistent organic pollutants and prenatal distress, which were not captured in the reported findings.

While gene expression levels were measured in the placenta, metal levels were measured in maternal toenails. This is a pertinent consideration as agreement between placental and maternal toenail levels were previously reported for some but not all assessed metals (Punshon et al. 2016). The observed discrepancies between placental and toenail metal levels can arise due to several reasons. For example, despite extensive washing steps in the processing of the toenails prior to ICP-MS analysis, exogenous metal sources may contribute to toenail measurements.

In addition to biospecimen differences, assessments in maternal vs. fetal exposures can differ due to differences in bioavailability. For example, some metals not only readily cross the placental barrier but also accumulate in placental tissue while others (for example, cadmium) cross only partially or not at all. The bioavailability of metals in the placenta can also vary based on inter-individual differences in toxicokinetics, including genetically-determined placental metabolic and transport activity (Gundacker et al. 2016). Hence, maternal toenail metal levels may not accurately reflect exposure levels directly experienced by the placenta. Instead, changes in placental activity may capture the combination of metal-induced toxicity within the placenta as well as metal-induced changes in maternal physiology, resulting, for example, in altered uteroplacental blood flow and secretion of maternal hormones, which ultimately lead to altered fetoplacental development.

Metal exposure was assessed in toenails collected postpartum, following the outcome assessment of birth weight. Still, given the slow growth rate of toenails (1mm/month), these postpartum measurements largely capture an integrated exposure measure spanning pregnancy (Garland et al. 1993; Yaemsiri et al. 2010). Hence, the detected changes in placental expression patterns are likely in response to constitutive rather than acute exposure. However, as both gene expression and metal exposure levels were measured at a single time point at term, the temporal dynamics between metal-induced changes in gene expression levels to tease out potential windows of susceptibility could not be further evaluated. Furthermore, since exposure and expression levels were determined contemporaneously with the outcome, the directionality of the association between these metrics and the outcome could not be established.

While our findings point to a possible convergence between *in utero* metal exposures, alterations in placental processes and deviations in fetal growth, we did not formally test for mediation in the current study due to limited sample size. Similarly, while we adjusted our models for potential confounding due to gender, we were underpowered to assess potential gender-specific effect modification in the associations between metal exposure and placental gene expression as well as SGA status. This is a particularly pertinent consideration given that As and Cd may act as endocrine disruptors (Iavicoli et al. 2009). Follow up studies with sufficient power to formally investigate whether changes in placental processes mediate the association between *in utero* metal exposures and SGA status and potential gender-specific effects are warranted to further evaluate the proposed pathway.

Finally, we were able to verify some, but not all, observed significant associations through technical replication in an independent cohort. The three cadmium-responsive genes selected for qRTPCR validation across the NHBCS cohort were prioritized based on a preliminary study on a subset of the cohort. Further efforts will need to be undertaken to more comprehensively assess the generalizability of the reported findings, especially with respect to the As-related findings.

Nevertheless, the findings reported in the current study provide an important advancement in the understanding of metal-induced changes in placental processes that disrupt appropriate fetal growth.

Conclusions

Leveraging placental transcriptomic and multi-metal exposure data, we delineate potential placental processes through which trace metal exposure impact fetal growth. The application of novel statistical and bioinformatics-based approaches that account for interrelationships within each dataset and facilitate integration across datasets informed the insight into pathways linking intrauterine trace metal exposures and placental processes relevant to perturbations in fetal growth reported in the current study. Implementation of such integrative approaches has the potential to advance mechanistic insight across the spectrum of exposure-disease paradigms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

This work is supported by NIH-NIMH R01MH094609, NIH-NIEHS R01ES022223, NIH-NIEHS R01ES022223-03S, NIH-NIEHS P01ES022832, NIH-NIEHS P42ES007373, NIH-NIEHS P30ES023515, NIH-NIEHS R24ES028507, NIH-NIGMS P20GM104416, and US-EPA RD83544201.

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Highlights

SGA status is associated with multi-metal index predominated by arsenic and cadmium

Two placental gene modules are associated with SGA status and multi-metal index

Gene modules are enriched for gene expression and metabolic hormone processes

Figure 1. Adjusted odds ratios and 95% CI for SGA status given a log unit increase in individual trace metal levels.

Borderline positive associations were observed between SGA status and As and Cd levels.

An inverse association was observed between SGA status and Ni levels.

Figure 2. Association between metal composite levels and SGA status based on weighted quantile sum (WQS) regression analysis.

Two separate WQS indices were generated, one modeled in the positive direction and one modeled in the inverse direction with respect to SGA status. Adjusted logistic regression models revealed that a WQS index predominated by contributions from As and Cd associated with increased odds of SGA status and a WQS index predominated by Ni and Al was associated with decreased odds of SGA status.

Figure 3. Association between placenta gene network modules and SGA status. Eigengene values of previously derived placental gene network modules were modeled against SGA status using covariate-adjusted logistic regression models. The gene expression (greenyellow) module is observed to be inversely associated with SGA status and the metabolic hormone secretion (salmon) module is observed to be positively associated with SGA status.

Figure 4. Association between placental gene network modules and metal exposure.

Among the SGA associated modules, covariate-adjusted models revealed a borderline positive association between the metabolic hormone secretion (salmon) module and Cd exposure as well as the SGA-associated WQS index, and an inverse association between the gene expression (greenyellow) module and As exposure as well as the SGA-associated WQS index.

Figure 6. qRT-PCR validation of the association between placental expression of metabolic hormone secretion (salmon) module genes and maternal Cd exposure in the NHBCS cohort. Covariate-adjusted generalized linear models revealed a positive relationship between gene expression and maternal Cd levels in the NHBCS, with the associations reaching statistical significance for *GRHL1* and *INHBA*. These findings are consistent with observations in the RICHs cohort.

Table 1.

Demographic and gestational characteristics of the RICHS study population (n=195)

Variables	SGA (n=43)	AGA (n=152)	p-value ^a
	<i>mean ± SD</i>	<i>mean ± SD</i>	
Birth weight (g)	2577.2 ± 268.4	3447.4 ± 376.3	<0.01
Gestational age (weeks)	38.9 ± 1.1	39.0 ± 1.0	0.94
Maternal age (years)	32.0 ± 5.3	31.4 ± 4.7	0.49
Maternal BMI (kg/m ²)	25.4 ± 6.7	26.0 ± 5.9	0.17
	<i>n (%)</i>	<i>n (%)</i>	
Infant Gender			0.10
Female	28 (65.1)	75 (49.3)	
Male	15 (34.9)	77 (50.7)	
Delivery Method			0.73
Vaginal	23 (53.5)	88 (57.9)	
Cesarean	20 (46.5)	64 (42.1)	
Maternal parity			0.24
0	21 (48.8)	58 (38.1)	
1	17 (39.6)	60 (39.5)	
2+	5 (11.6)	34 (22.4)	
Maternal Ethnicity			<0.01
Caucasian	25 (58.1)	122 (80.3)	
African American	7 (16.3)	3 (2.0)	
Other	9 (20.9)	26 (17.1)	
Unknown	2 (4.7)	1 (0.6)	
Maternal Education			0.91
High School or less	6 (13.9)	19 (12.5)	
Some College	10 (23.3)	32 (21.0)	
College Graduate	27 (62.8)	100 (65.8)	
Unknown	0 (0)	1 (0.7)	
Maternal Smoke Status			0.04
Yes	3 (7.0)	1 (0.6)	
No	38 (88.4)	148 (97.4)	
Unknown	2 (4.6)	3 (2.0)	

^a p-values comparing SGA and AGA infants based on Mann-Whitney U test for continuous variables and ChiSquare test for categorical variables

Table 2.

Maternal metal levels (ug/g dry weight toenail) in RICHs

Metal	Mean	Standard Deviation	Minimum	Maximum
Al	19.004	40.505	1.595	532.579
As	0.049	0.045	0.011	0.445
Cd	0.016	0.019	0.001	0.170
Cr	0.386	0.492	0.033	3.408
Cu	23.137	60.159	0.241	717.064
Fe	18.196	19.537	3.504	220.161
Mn	0.265	0.742	0.029	11.209
Mo	0.018	0.032	0.002	0.366
Ni	20.064	61.330	0.039	525.445
Pb	0.315	0.541	0.002	5.124
Sb	0.038	0.064	0.002	0.676
Se	0.979	0.241	0.031	3.132
Sn	0.286	0.374	0.004	3.198
U	0.007	0.020	0.000	0.192
V	0.029	0.055	0.002	0.568
Zn	102.290	17.751	39.086	164.630